COMPETITION AMONG THE AMINOBENZOATE IONS AND THE METHYL RED IONS FOR BINDING SITES ON BOVINE SERUM ALBUMIN

by

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21 Table 5: Numerical figures in the third and fourth column should be multiplied by the factor 0.8 to obtain the correct value.

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INTRODUCTION

The binding of organic ions with proteins has been observed by many investigators. It has been found in general that structural changes in a dye molecule can make large changes in its ability to bind with a protein. It has been noted also that if the protein is modified there are marked changes in the protein-dye complexes that are formed.

Early workers have noted that wool and casein exhibited selective binding of dextro mandelic acid from a racemic mixture (Bradley and Easty, 4). More recently Karush (9) has shown that bovine serum albumin binds the optically active dyes and that it exhibits some selectivity for different forms. Similarly other investigators have shown that the different position isomers exhibited different binding abilities with proteins (Luck and Schmit, 16).

Klotz and co-workers (11, 14, 15) have shown that differently charged anionic dye molecules disclosed distinctive binding abilities with bovine serum albumin (BSA). They have also shown that methyl orange, among other dyes investigated, also formed complexes with bovine serum albumin. It was shown that the protein-dye complexes were accompanied with a decrease in a maximum absorbency index and in some cases a shift in the wavelength of the absorption peak. It was shown also that the addition of any one of the aminobenzoic acids to a solution of the protein-dye complex will reverse the effect of the protein on the spectrum of the dye. Such a displacement is thought to envolve a competitive action of the carboxylate ions and the dye for the binding sites on the protein molecule. Klotz (11) has shown that the ortho-aminobenzoate ion is a better competitor than the para isomer

in displacing the methyl orange of the protein-dye complex. The study of the protein interactions of the ortho and para isomers of the aminobenzoate ions was confirmed by Burkhard (3) and extended by him to include the meta aminobenzoate ion. It was noted that the meta isomer had no greater affect on the displacement of methyl orange than the para isomer. Further studies involving the methyl reds (Klotz et al., 12) have shown that the meta isomer binds more strongly than "methyl red" (ortho isomer) which in turn binds stronger than its para isomer.

It has been indicated that the ions derived from meta and para aminobensoic acids cannot very well engage in intramolecular hydrogen bonding as the ortho isomer. This leaves the results obtained with the aminobenzoate isomers to be discussed in terms of their acid strengths and molecular configuration.

It has been the purpose of this research to determine if there is some relationship between the binding ability of the aminobenzoate ions and the methyl reds for the binding sites on bovine serum albumin and hence gain an insight into the factors which determine the ability of an anion to complex with this protein.

EXPERIMENTAL

Preparation of Dyes

4-Dimethylaminoazobenzene-2'-carboxylic acid ("methyl red"). This dye was made according to the procedure outlined in Gilman and Blatt (7). The dye was recrystallized twice from hot ethyl alcohol and then dried in an oven at 110°C for one hour.

Observed melting point, 179.5 - 180°C Uncorrected.

Literature, 181 - 182°C (7) Uncorrected.

4-Dimethylaminoazobenzene-3'-carboxylic acid (Theil, 19). This dye was prepared according to the procedure outlined for the ortho isomer. The same experimental conditions were used. The isolated dye was recrystallized twice from absolute ethyl alcohol and then dried in an oven at 110°C for one hour.

Observed melting point was 199°C Uncorrected.

Literature, 210°C (19) Corrected.

4-Dimethylaminoazobenzene-4'-carboxylic acid. This dye was prepared according to the procedure outlined by Burkhard (2). The para-methyl red prepared in this way crystallized from a pyridine-water mixture and was reported to have a melting point of 271-273°C.

There was considerable difficulty in obtaining a product by this above procedure with the desired melting point. Preparation of the para-methyl red by the above procedure was again repeated. Successive recrystallizations from mesityl oxide gave a melting point of 216-216.3° C. A sample was prepared for chemical analysis by recrystallizing it from mesityl oxide and drying it in a drying pistol over phosphorous pentoxide.

Calculated nitrogen	15.61 percent
Found[1]	15.10 percent

The melting point of the dye prepared in this manner did not agree with that prepared by Burkhard. A spectra of the dye crystallized from mesityl oxide and Burkhard's sample showed a slight difference in the ultra-violet region. This difference was suspected to be due to pyridine present. Both of these dyes were compared to a sample of the dye obtained from the Eastman Kodak Company. Eastman's sample crystallized from mesityl oxide, Burkhard's sample, and a sample of the dye crystallized from mesityl oxide were placed in a vacuum oven at 150°C and 30 mm pressure for eight hours. The observed melting points for all samples were 245°C uncorrected. A mixed melting point melted at 245°C which apparently is the true melting point and not the two previously reported. It appears that both samples were contaminated with the crystallizing solvent but this apparently had no effects on the results obtained in the binding experiments.

Preparation of Buffers

The buffer that was employed throughout these experiments was made to have a pH value of 6.8. The buffer was prepared by weighing out 7.87 g of disodium hydrogen phosphate and 6.0 g of potassium dihydrogen phosphate and diluting to one liter. The buffer solution was checked

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[1]Analytical data supplied by Drs. G. Weiler and F. B. Strauss, 164 Banbury Road, Oxford, England.
A 2.780 mg sample gave 0.364 mls of nitrogen at a pressure of 761 mm of mercury at 22°C and thus calculated to give 15.1 percent nitrogen content in the sample.

with a standard buffer solution and, when necessary, corrected accordingly.

Determination of Dry Weight of Protein

A small sample of the protein was weighed out in a previously weighed crucible. The protein was dried in an oven at 110°C overnight and then cooled and weighed. This was repeated until a constant value was obtained. The loss in weight represents the water content of the protein and consequently the percent of dry proteinaceous material present can be calculated. The crystallized bovine serum albumin was obtained from the Armour Laboratories and Lot No. M66909 employed in these studies contained 96.51 percent proteinaceous material.

Determination of Absorption Spectra

Ortho- and Meta-Methyl Reds. The determination of the spectra of the ortho- and meta-methyl reds was made as follows. An appropriate amount of the dye was weighed out and transferred to a 100 ml volumetric flask. The sample was then dissolved in absolute ethyl alcohol and cooled in a bath to 20°C and filled to the mark. A 10 ml sample was pipetted to another 100 ml volumetric flask and the alcohol evaporated off under reduced pressure. A fine capillary tube was inserted deep into the flask and suction continued for ten minutes to make sure that all the ethyl alcohol was removed. Phosphate buffer was then added to the flask containing a known amount of the dye. The solution was warmed on a steam bath whenever it was necessary to get the dye in solution and then cooled to room temperature and filled to the mark.

For a normal spectrum, the buffer was placed in one cell as a blank and the solution of the dye in buffer in the other cell. The spectrum was scanned between 320 to 500 mμ. The wavelength of maximum absorption was then determined upon both solutions.

Para-Methyl Red. The absorption spectra for this dye was determined in the same manner as the ortho- and meta-methyl reds were except the dye was dissolved in a 250 ml volumetric flask with absolute ethyl alcohol. Fifteen ml aliquot portions were then transferred to a 250 ml volumetric flask and the solvent evaporated. The residual sample was then dissolved in the buffer and the spectrum recorded.

The Beckman Model D. U. spectrophotometer was employed for all spectral work. The one centimeter corex cells were used in the visible region and one centimeter quartz cells for spectral observations in the ultra-violet. Constant maximum sensitivity was maintained and the slit width was varied to cause the needle to return to the zero point. In this manner the minimum slit width was used at all wavelengths.

The molar absorbancy index was determined by transferring aliquot alcoholic samples of the dye to volumetric flasks. The solvent was then evaporated and replaced with buffer. The optical densities of the dye in buffer were recorded. Since the Beer-Lambert Law holds, the optical densities can be converted into molar absorbancy index or concentrations by utilizing the following equation:

$$\text{Optical Density} = \log I_0/I = \epsilon\, c\, l$$

Where I_0 is the intensity of the incident light, I is the intensity of the transmitted light, l is the length of the light path in centimeters, c is the concentration in moles per liter and ϵ is the molar absorbancy index. This method allowed calculations of the molar absorbancy index

of the methyl reds in phosphate buffers to be made.

Protein Complexes. The spectra of the dye, aminobenzoate ions and its protein-dye complex were determined as follows. Stock solutions of the dyes were made up to a concentration of 3×10^{-5} M/L, the protein and aminobenzoic acids concentrations were made up to 0.6 percent. For the normal spectra of the dye, 10 ml of the stock dye was placed in a test tube and 20 ml of buffer was added. This solution was shaken thoroughly and placed in one cell. Buffer solution was placed in the other cell as a blank.

For the protein-dye complex, 10 ml samples of protein solution was placed in two test tubes. Ten ml of dye solution was placed in one. To the first tube 10 ml of buffer and to the second tube 20 ml of buffer solution was added. These solutions were well shaken to assure a homogenous mixture. The protein-buffer solution served as a blank for the protein-dye complex. The spectrum thus obtained was that of the protein-dye complex.

For the protein-dye-aminobenzoate ion complex, 10 ml sample of protein stock solution was placed in two test tubes. To each of these tubes 10 ml of the aminobenzoic acid solution corresponding to the dye involved was added. Ten ml of dye stock solution was added to one tube and 10 ml of buffer to the other. Both were well shaken. The protein-aminobenzoate ion solution was used as a blank for the protein-dye-aminobenzoate ion solution. The spectrum thus obtained was that of the protein-dye-aminobenzoate ion complex. The resulting solution contained concentrations of the dye equal to 1×10^{-5} M/L, the protein equal to 0.2 percent and the aminobenzoate ion 0.2 percent determined as amino-

benzoic acid. This procedure was used with both the ortho- and meta-methyl reds. However, the para-methyl red was less soluble and a stock solution having a concentration of 2×10^{-5} M/L could be made. Appropriate dilutions were then employed to give a solution of the same concentrations.

Binding Technique

Quantitative determinations of the amount of dye or aminobenzoate ion bound was determined by the equilibrium-dialysis technique. A 0.2 percent solution of protein to be bound was placed in a cellulose bag and immersed in the dye solution. The tubes containing the bag and dye were shaken from 12 to 18 hours in a constant temperature bath at 0°C. The bag was removed and the concentration of the dye outside the bag was determined spectrophotometrically. The concentration of the dye was determined from the previously determined molar absorbancy index and then applying the Beer-Lambert Law. The amount of dye outside the bag permits calculations of the amount of dye bound by the protein.

The following is an example of a binding run. The cellulose strips to be used were soaked in several changes of water for at least three hours. Each cellulose strip was cut to a length of 8-10 inches. The protein solution containing .080 g of bovine serum albumin and 40 ml of buffer was prepared and mixed thoroughly. The dye or aminobenzoate ion solution was prepared by proper dilution of a stock solution. Twenty ml of buffer or dye or aminobenzoate ion was then pipetted into a large test tube. A casing was then removed from the water and stripped between two fingers to remove the maximum amount of water. A knot was tied in the

casing to make sure no solution escaped from the bag, and then five ml of protein solution was pipetted into the bag. The protein solution was compressed into as small a volume as possible and the bag was sealed with two knots. The excess casing at each end of the bag was cut off about 0.5 inches from the knots. A piece of white cotton thread was tied to one end between the knots. The loose end was cut about 0.5 inches from the knot. The bag was then suspended in the test tube with the other end of the thread jammed between the rubber stopper and the tube.

For each bag prepared with protein another one was prepared with buffer only. Both bags were placed in tubes containing the same concentration of the dye and/or aminobenzoate ion.

The test tubes were then placed on the Burrell shaker in a constant temperature bath and shaken for a period of 12-18 hours. The bags were then removed and the concentration of the dye was determined by means of the spectrophotometer. The tube which had the buffer on the outside and the protein bag served as a blank for all those tubes which contained the protein bag. In a similar way, the tube which had buffer on the outside and the buffer bag served as a blank for all those tubes containing the buffer bag. The optical densities were taken at the absorption maximum for the particular dye used, and, from the previously expressed relationship of the Beer-Lambert Law, the final concentrations of dye and protein were determined.

The amount of dye or aminobenzoate ion bound was then determined by subtracting the concentration of the dye or aminobenzoate ion in the protein bag tube from the concentration of the dye or aminobenzoate ion in the buffer bag tube. The data was then expressed by the quantity r

(the moles of anion bound per mole by protein) which was found by dividing the amount of dye or aminobenzoate ion bound by the amount of protein present in five ml of 0.2 percent protein solution.

The dye could be measured spectrophotometrically directly. However, the aminobenzoate ion had to be converted to its corresponding Schiff's base with p-dimethylaminobenzaldehyde and its resultant color measured directly as described below.

Determination of Aminobenzoic Acids

Quantitative determinations of the aminobenzoate ions were made by employing the technique of Rindi (18). One gram of p-dimethylaminobenzaldehyde was dissolved in 50 ml of a 25 percent acetic acid solution. This was diluted to 100 ml. Ten ml of this stock solution was transferred with a pipette to a 100 ml volumetric flask and filled to the mark with 10 percent acetic acid solution. The concentration of the resulting reagent is 0.1 percent. The absorption maximum was determined for each base formed with the aminobenzoate ion and p-dimethylaminobenzaldehyde. The compound formed from the ortho isomer and para isomer absorbed at 450 mμ and that from the meta isomer at 440 mμ. A calibration curve was drawn based upon absorption data obtained for each of the aminobenzoate ions each time they were determined. The intensity of color developed was found to vary with the concentration of aminobenzoate ion and to obey Beer's Law.

The following is an example of an aminobenzoate ion determination. Fifteen ml of a solution containing aminobenzoic acid in buffer was transferred from a test tube after binding was completed to a clean test

tube. To this was added 3 ml of a 0.1 percent p-dimethylaminobenzaldehyde reagent. A yellow color developed within five minutes. The solution was thoroughly shaken. Twenty minutes after mixing with the reagent the optical density was recorded at the maximum absorption for the particular aminobenzoate ion involved. The volumes for all determinations were constant. For the calibration curve, 15 ml solution of a .0008 percent concentration of aminobenzoic acid in buffer was used as the first point. Then a 10 ml sample of the .0008 percent concentration of aminobenzoic acid and 5 ml of buffer for the second point. For the third point 5 ml of .0008 percent concentration of aminobenzoic acid and 10 ml of buffer. For each point 3 ml of 0.1 percent p-dimethylaminobenzaldehyde reagent was added to develop the color. The calibration was determined by plotting optical densities versus volume of .0008 percent aminobenzoic acid solution used.

RESULTS AND DISCUSSION

Klotz and Walker (14) have shown that the spectrum of azosulfathiazole among other dyes studied, is affected by the addition of protein bovine serum albumin. It has been shown by them that this change was due largely to an interaction between the protein and the dye. The alteration of the dye spectrum was shown not to be affected by a slight change in pH by the addition of the protein. They showed also that this shift was not due to displacements in dimer or polymer equilibria as the dye was in the mono-meric state in aqueous solution. Klotz concludes that "the interaction of the bovine serum albumin and azosulfathiazole is a specific effect due to the combination of the protein with the dye anion to form

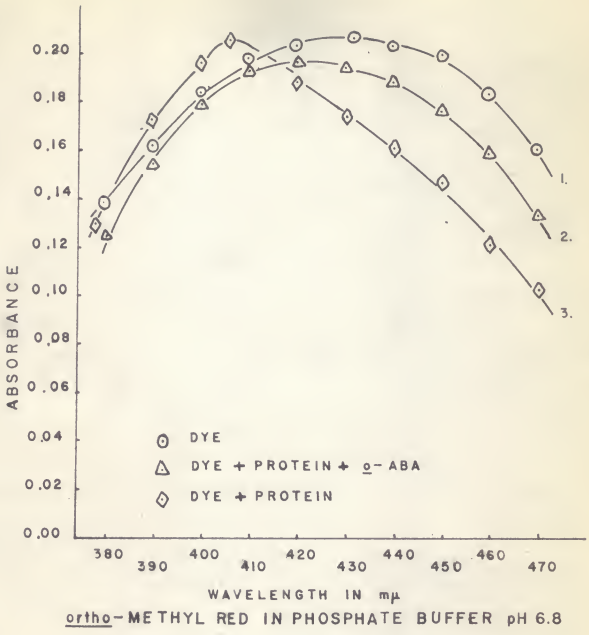
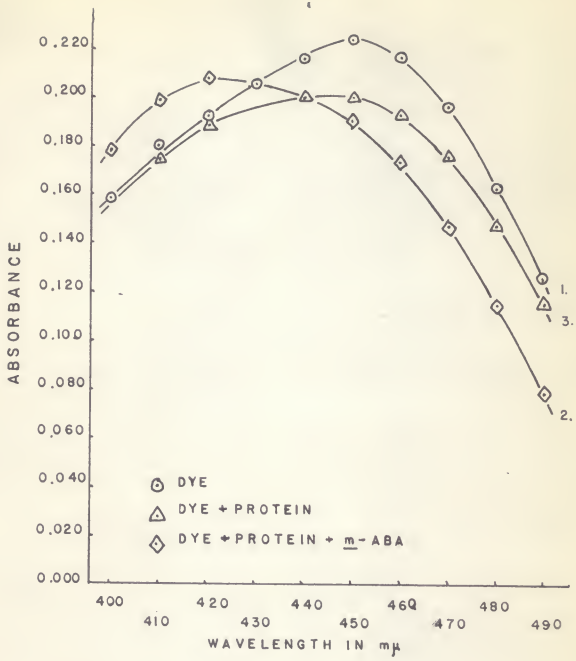
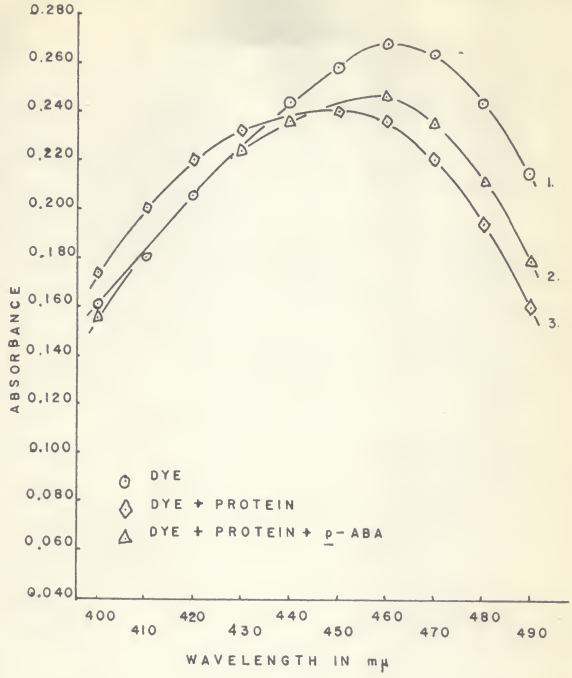


Fig. 1. Spectra of systems involving the ortho-methyl red.



meta-METHYL RED IN PHOSPHATE BUFFER pH 6.8

Fig. 2. Spectra of systems involving the meta-methyl red.



para—METHYL RED IN PHOSPHATE BUFFER pH 6.8

Fig. 3. Spectra of systems involving the para-methyl reds.

an intramolecular complex."

The addition of bovine serum albumin to a buffered solution of the methyl reds alters their spectrum. This result is evidenced when curve 3 is compared with curve 1 of ortho-methyl red in Fig. 1. This same alteration was noticed for meta-methyl red (Fig. 2) and para-methyl red (Fig. 3). However, the degree of displacement was greater for ortho-methyl red-protein complex than for the para-methyl red-protein complex and to a lesser degree the meta-methyl red-protein complex. The order of the degree of displacement could not in any way be attributed to the order of degree of binding. Changes in the absorbancy likewise did not parallel the order of binding (Table 1).

Table 1. Change in absorbancy of the dyes and their complexes.

System	Absorbancy	Change in Absorbancy
at 432 mu		
o-MR*	(.206)	0
o-MR + BSA*	(.173)	.033
o-MR + BSA + o-ABA*	(.193)	.013
at 450 mu		
m-MR*	(.223)	0
m-MR + BSA	(.200)	.023
m-MR + BSA + m-ABA*	(.191)	.032
at 460 mu		
p-MR*	(.268)	0
p-MR + BSA	(.236)	.032
p-MR + BSA + p-ABA*	(.246)	.022

*Abbreviations: o-MR, ortho-methyl red; m-MR, meta-methyl red; p-MR, para-methyl red; BSA, bovine serum albumin; o-ABA, ortho-aminobensoic acid; m-ABA, meta-aminobenzoic acid; p-ABA, para-aminobenzoic acid.

Concentrations employed: all dye solutions were 1×10^{-5} molar, protein solutions were 0.2 percent, and aminobenzoic acid solutions were 0.2 percent.

The methyl reds were found to obey Beer's Law and from this it could be concluded that micelle does not take place. The observations made are due to a protein interaction. Spectral properties of the dyes and their complexes are given in Table 2.

Table 2. Spectral properties of dyes and their complexes in phosphate buffer pH 6.8.

System	Wavelength of Maximum Absorption (mμ)	Molar Absorbancy Index x 10^{-3}
o-MR*	432	20.4 ± .1
o-MR + BSA*	406	20.0
o-MR + BSA + o-ABA*	420	19.4
m-MR*	450	22.3 ± .1
m-MR + BSA	448	20.1
m-MR + BSA + m-ABA*	420	20.8
p-MR*	460	26.6 ± .1
p-MR + BSA	451	23.8
p-MR + BSA + p-ABA*	460	24.4

*Abbreviations: same as in Table 1.
Concentrations: same as in Table 1.

It has been stated earlier that Klotz and Burkhard studied the competitive action of the aminobenzoate ions with methyl orange, and found that the ion from ortho-aminobenzoic acid was a better competitor than either of the ions from meta- and para-aminobenzoic acids. It was also noted that the latter two were of about equal in their ability to displace the methyl orange from its protein complex.

A competitive action of the aminobenzoate ions was studied with the methyl reds with the following results obtained for the best competitive system (Table 3).

Table 3. Amounts of dye bound with and without competitors (r) in phosphate buffer pH 6.8.

Dye	No Competitor	Competitors		
		o-ABA*	m-ABA*	p-ABA*
o-MR* + BSA*	0.682	0.175	0.306	0.245
m-MR* + BSA	0.769	0.568	0.432	0.320
	0.747	0.600	0.433	0.368
p-MR* + BSA	0.248	0.114	0.114	0.114

*Abbreviations: same as in Table 1.
Concentrations: same as in Table 1.

For the ortho-methyl red-protein complexes the ortho-aminobenzoate ion was the best competitor followed by the para-aminobenzoate ion and then the meta-aminobenzoate ion. For the meta-methyl red-protein complex the para-aminobenzoate ion was the best competitor followed by the meta-aminobenzoate ion and to the other extreme the ortho-aminobenzoate ion. For the para-methyl red-protein complex all three aminobenzoate ions were of equal strength in their competitive ability.

It has been pointed out that the spectrum of the ortho-methyl red and para-methyl red is shifted by the addition of bovine serum albumin. When a competitive action is undertaken with the aminobenzoate analog of the corresponding methyl reds there is a tendency for the spectrum to shift towards its normal absorption wavelength. A somewhat reverse effect was noted with meta-methyl red. The dye-protein spectrum was not appreciably shifted to begin with, but, when the corresponding aminobenzoate ion was added, a drastic shift of the spectrum was noted (Figs. 1, 2, and 3). This would seem to indicate that the meta-methyl red has a preference for different binding sites on bovine serum albumin than

either of its corresponding isomers.

The quantitative determination of the extents of binding of each of the aminobenzoate ions using the p-dimethylaminobenzaldehyde reagent were made at the wavelengths for maximum absorption of the resulting Schiff's bases. (Table 4)

Table 4. Spectral properties of the resulting Schiff's bases.

System	Wavelength of Maximum Absorption (mμ)
o-ABA* + p-DMAB*	450 mμ
m-ABA + p-DMAB	440 mμ
p-ABA + p-DMAB	450 mμ

*Abbreviations: same as in Table 1, plus p-DMAB, para-dimethylaminobenzaldehyde.

A time dependency on color development for each aminobenzoic acid with the aldehyde reagent is shown in Fig. 4. The curves show a blooming effect which becomes steady after standing for fifteen minutes and is shown to be steady for an additional fifteen minutes. The readings were made after a lapse of twenty minutes subsequent to mixing with the aldehyde reagent. A calibration curve for the quantitative determination for each of the aminobenzoic acids is shown in Fig. 5.

Results of the binding by BSA revealed that the ortho-aminobenzoate ion was bound in largest quantity followed by meta- and para-aminobenzoate ions (Table 5).

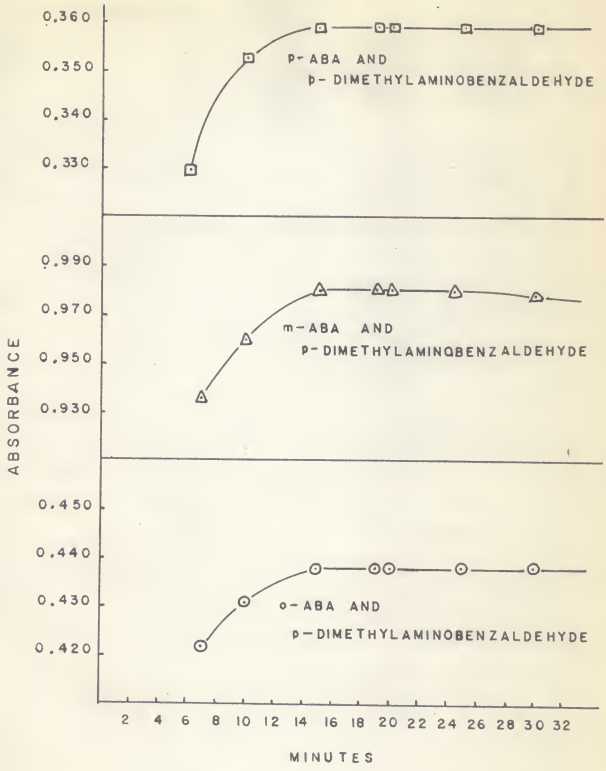


Fig. 4. Stability of colored Schiff's bases of amino-benzoic acids and para-dimethylaminobenzaldehyde.

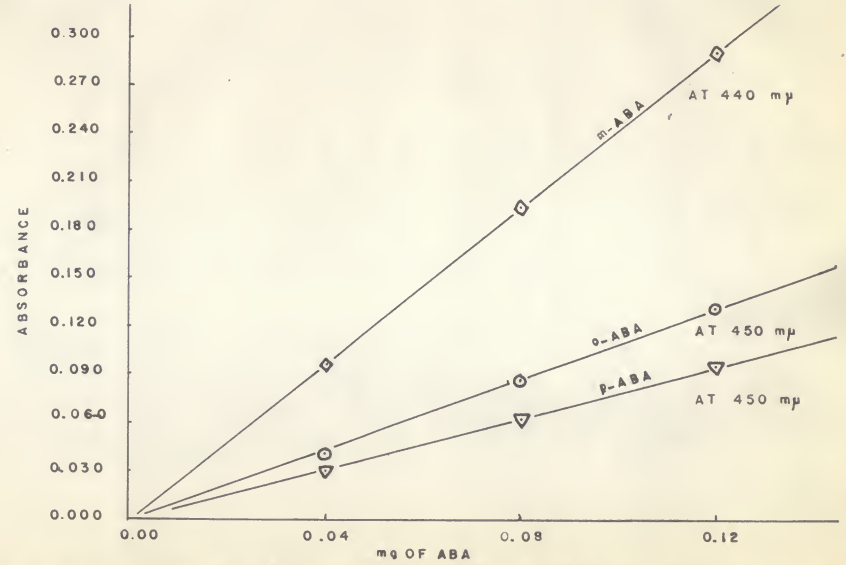


Fig. 5. The relationship of the absorbance and concentrations of the Schiff's bases of aminobenzoic acids and para-dimethylaminobenzaldehyde.

Table 5. Mean value of quantity (the moles of aminobenzoate ion bound per mole by protein),

System	Number of Determinations	Mean r Value	Standard Deviation
o-ABA* + BSA*	17	1.1	.30
m-ABA* + BSA	12	.628	.088
p-ABA* + BSA	12	.47	.18

*Abbreviations: same as in Table 1.

The significance of these results will be discussed later.

Since an increase in the molecular weight (Burgert, 1) of an anion as well as other factors, pH of the solution, etc. can affect the extent of binding by a protein, and if one retains these factors constant, then perhaps a comparison can be made with reference to a group of anions whose members differ only in configuration. Such a group of anions were chosen for these studies; methyl red and its corresponding isomers.

If one were to make comparative models of the methyl reds (from Fisher-Taylor-Hirschfelder models), then they would suggest that ortho-methyl red binding through the $-COO^-$ would be stearicly hindered by its configuration and that it would favor a "specie" binding site which would be more apt to fit to its configurational demands.

It also appears from these models that since the meta- and para-methyl reds will have different configurations, they in turn may also require different "specie" binding sites.

The spectra of the binding of the methyl reds indicate that such "specie" binding sites may be in order (Figs. 1, 2, and 3). Furthermore, for ortho-methyl red the aminobenzoate ions competed for the binding site in the following order:

o-ABA > p-ABA > m-ABA

For meta-methyl red the order was:

p-ABA > m-ABA > o-ABA

For para-methyl red all of the aminobenzoate ions competed equally for the binding site.

The reverse affect of the spectral shift of meta-methyl red (Fig. 2) in the presence of protein alone, and in the presence of protein and the aminobenzoate analogs, compared to the spectrum shifts of the remaining methyl red isomers so treated (Figs. 1 and 3) leads us to believe that meta-methyl red has a "specie" site specificity which differs from that of the ortho- or para-methyl red compounds.

The degree of binding of the methyl reds with bovine serum albumin is directly related to their K_a (Burkhard, 2) values. That is, the K_a value of the meta isomer is greater than the ortho isomer, which, in turn, is greater than its para isomer.

The trend of the binding of the aminobenzoic acids with bovine serum albumin is ortho, meta, and para. This would not seem to be their proper order if such binding was related to their K_a (Cumming, 6 - Johnston, 8) values. Their order of K_a values is meta, para, ortho. However, models of a peptide linkage and the ortho-aminobenzoate ion configuration were constructed. These models offer a strong suggestion that there is a "lock and key" effect with hydrogen bonds envolving the >N-H and >C=O of the peptide linkage, and the COO^- and NH_2 groups of ortho-aminobenzoate ion. Thus, the ortho-aminobenzoate ion could be bound in a radically different fashion than the other two aminobenzoate ions.

However the ortho-methyl red is bound it is apparent that it is bound differently than its meta-methyl red isomer. This appears to be evident since in the former ortho-aminobenzoic acid is a better competitor than its other isomers, whereas in the latter case it is the poorest competitor.

As for the para-methyl red, its binding site is not too clear since all of the aminobenzoic acid isomers are equally good as competitors.

The significance of the para- and meta-aminobenzoic acids as competitors of the methyl reds for binding sites is not very clear. It was thought in the case of the meta-aminobenzoic acid that the amino group would play a significant part as a competitor. However, the present observations do not substantiate this viewpoint.

Solubility studies were made on ortho-methyl red, meta-methyl red and para-methyl red. The solubilities were found to be 9.60×10^{-5}, 5.36×10^{-4}, and 2.41×10^{-5} moles/liter respectively. The striking significance of these observations is the direct relationship between solubilities and extents of binding.

It is important to note that the order of decreasing solubility of the aminobenzoic acids is meta, ortho, and para (Cohn and Edsall, 5). Assuming that the ortho-aminobenzoic acid is enhanced by hydrogen bonding in its binding ability, then the order of the remaining two isomers parallels the same trend in the extent of their binding.

SUMMARY

The binding of the methyl reds and their aminobenzoate ion analogs by bovine serum albumin has been studied. The action of the aminobenzoate ions as competitors with the methyl reds for bovine serum albumin were also included.

Spectral variations of the dye-protein complex of the ortho- and para-methyl reds were found to be similar. However, a significant variation was noted for meta-methyl red. Although no definite conclusion can be made from competitive studies of the aminobenzoate ions regarding the type of binding that occurs, it is suggested that the ortho-methyl red is bound differently than the meta-methyl red. There is also an indication that the para-methyl red may also be bound differently than either of these two isomers.

The ortho-aminobenzoate ion was found to be bound by bovine serum albumin to a greater degree than either its meta isomer or para isomer.

ACKNOWLEDGMENT

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APPENDIX

SPECTRUM OF ortho-METHYL RED

Temp. 25°C. 19.28 mg in a liter of phosphate buffer solution, pH 6.8. 7.16 x 10^{-5} molar.

Wavelength	Absorbancy Index x 10^{-3}	Wavelength	Absorbancy Index x 10^{-3}
320 m. micron	3.81	432 m. micron	20.5
330	3.68	440	20.3
340	3.95	450	19.5
350	5.99	460	18.0
360	7.91	470	15.4
370	10.8	480	12.2
380	13.5	490	8.68
390	16.1	500	5.57
400	18.2	520	2.02
410	19.5	540	.826
420	20.2	560	.686
430	20.4	580	.224

SPECTRUM OF para-METHYL RED

Temp. 25°C. 13.50 mg in a liter of phosphate buffer solution, pH 6.8. 5.01 x 10^{-5} molar.

Wavelength	Absorbancy Index x 10^{-3}	Wavelength	Absorbancy Index x 10^{-3}
320 m. micron	3.70	430 m. micron	20.5
330	3.18	440	21.7
340	3.08	450	22.4
350	4.36	460	21.7
360	6.32	470	19.8
370	8.80	480	16.7
380	11.6	490	13.0
390	14.2	500	8.84
400	16.3	520	3.10
410	18.0	540	.680
420	19.5	560	.080

SPECTRUM OF para-METHYL RED

Temp. 25°C. 1.46 mg in a liter of phophate buffer solution, pH 6.8. 5.42 x 10^{-6} molar.

Wavelength	Absorbancy Index x 10^{-3}	Wavelength	Absorbancy Index x 10^{-3}
320 m. micron	9.44	430 m. micron	23.5
330	8.14	440	24.8
340	7.22	450	25.7
350	8.14	460	26.5
360	9.62	470	26.1
370	11.3	480	24.2
380	13.7	490	21.1
390	16.3	500	17.2
400	18.3	520	9.44
410	20.4	540	4.26
420	21.8	560	2.40

Binding of o-methyl red with and without competitors. pH 6.8. 0°C.

System	Tube	Conc. M/L x 10^{-8}	Amt. Bound M x 10^{-8}	Protein M x 10^{-8}	r
Dye	1	794			
	1a	784			
Dye + BSA	2	417	9.42	14.0	0.673
	2a	402	9.56	14.0	0.683
Dye + o-ABA	3	765			
	3a	765			
Dye + BSA + o-ABA	4	672	2.33	14.0	0.166
	4a	662	2.57	14.0	0.184
Dye + BSA + m-ABA	5	598	4.17	14.0	0.298
	5a	588	4.41	14.0	0.315
Dye + BSA + p-ABA	6	632	3.31	14.0	0.236
	6a	622	3.56	14.0	0.254

Binding of p-methyl red with and without competitors. pH 6.8. 0°C.

System	Tube	Conc. M/L x 10^{-8}	Amt. Bound M x 10^{-8}	Protein M x 10^{-8}	r
Dye	1	668			
	1a	659			
Dye + BSA	2	233	10.9	14.0	0.777
	2a	233	10.6	14.0	0.761
Dye + m-ABA	3	673			
	3a	673			
Dye + BSA + o-ABA	4	350	8.07	14.0	0.576
	4a	354	7.96	14.0	0.568
Dye + BSA + m-ABA	5	422	6.28	14.0	0.448
	5a	440	5.83	14.0	0.416
Dye + BSA + p-ABA	6	498	4.37	14.0	0.312
	6a	489	4.60	14.0	0.328
Dye	7	682			
	7a	695			
Dye + BSA	8	274	10.2	14.0	0.729
	8a	265	10.8	14.0	0.769
Dye + m-ABA	9	713			
	9a	713			
Dye + BSA + o-ABA	10	386	8.18	14.0	0.584
	10a	372	8.52	14.0	0.609
Dye + BSA + m-ABA	11	471	6.06	14.0	0.432
	11a	471	6.06	14.0	0.432
Dye + BSA + p-ABA	12	511	5.04	14.0	0.360
	12a	502	5.27	14.0	0.376

Binding of p-methyl red with and without competitors. pH 6.8. 0°C.

System	Tube	Conc. M/L x 10^{-8}	Amt. Bound M x 10^{-8}	Protein M x 10^{-8}	r
Dye	1	605			
	1a	598			
Dye + BSA	2	459	3.67	14.0	0.262
	2a	466	3.29	14.0	0.235
Dye + p-ABA	3	620			
	3a	620			
Dye + BSA + o-ABA	4	553	1.69	14.0	0.121
	4a	560	1.50	14.0	0.107
Dye + BSA + m-ABA	5	553	1.69	14.0	0.121
	5a	560	1.50	14.0	0.107
Dye + BSA + p-ABA	6	553	1.69	14.0	0.121
	6a	560	1.50	14.0	0.107

Binding of p-aminobenzoic acid. pH 6.8. 0°C.*

Tube	O. D.	Percent Bound	Amt. Bound M x 10^{-8}	r
1	.065			
1a	.061	6.2	9.0	.64
2	.067			
2a	.061	9.0	13.	.93
3	.094			
3a	.078	17.	25.	1.8
4	.092			
4a	.080	13.	19.	1.4
5	.093			
5a	.080	14.	20.	1.5
6	.092			
6a	.081	12.	17.	1.2
7	.092			
7a	.079	14.	21.	1.5
8	.102			
8a	.092	9.8	14.	1.0
9	.102			
9a	.092	9.8	14.	1.0
10	.102			
10a	.094	7.8	11.	0.82
11	.099			
11a	.093	6.1	8.9	0.63
12	.104			
12a	.092	12.	17.	1.2
13	.102			
13a	.093	8.8	13.	0.92
14	.103			
14a	.092	11.	16.	1.1
15	.102			
15a	.093	8.8	13.	1.9

Tube	O. D.	Percent Bound	Amt. Bound M x 10^{-8}	r
16	.102			
16a	.092	9.8	14.	1.0
17	.100			
17a	.092	8.0	12.	0.83

*Total initial p-ABA present was 1.458×10^{-6} M.
Total amount of BSA present was 14.0×10^{-8} M.

Binding of p-aminobenzoic acid. pH 6.8. 0°C.*

Tube	O. D.	Percent Bound	Amt. Bound M x 10^{-8}	r
1	.139			
1a	.129	7.12	10.4	0.741
2	.227			
2a	.218	3.96	5.77	0.412
3	.229			
3a	.215	6.11	8.91	0.636
4	.231			
4a	.216	6.49	9.46	0.676
5	.231			
5a	.217	6.06	8.84	0.631
6	.231			
6a	.217	6.06	8.84	0.631
7	.228			
7a	.217	4.82	7.03	0.502
8	.232			
8a	.217	6.47	9.43	0.674
9	.231			
9a	.217	6.06	8.84	0.631
10	.232			
10a	.217	6.47	9.43	0.674
11	.232			
11a	.217	6.47	9.43	0.674
12	.231			
12a	.217	6.06	8.84	0.631

*Total initial p-ABA present was 1.458×10^{-6}M
Total amount of BSA present was 14.0×10^{-8}M

Binding of p-aminobenzoic acid. pH 6.8. 0°C.*

Tube	O. D.	Percent Bound	Amt. Bound M x 10^{-8}	r
1	.076			
1a	.076	7.9	12.	0.82
2	.077			
2a	.071	7.8	11.	0.81
3	.076			
3a	.073	4.0	5.8	.41
4	.074			
4a	.072	2.7	3.9	.28
5	.076			
5a	.073	4.0	5.8	41
6	.076			
6a	.073	4.0	5.8	.41
7	.077			
7a	.075	2.6	3.8	.27
8	.076			
8a	.073	4.0	5.8	.41
9	.077			
9a	.074	4.0	5.7	.41
10	.076			
10a	.072	5.3	7.7	0.55
11	.077			
11a	.073	5.2	7.6	0.54
12	.076			
12a	.074	2.6	3.8	0.27

*Total initial p-ABA present was 1.458×10^{-6} M.
Total amount of BSA present was 14.0×10^{-8} M.

COMPETITION AMONG THE AMINOBENZOATE IONS AND THE METHYL RED IONS FOR BINDING SITES ON BOVINE SERUM ALBUMIN

by

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The binding of organic ions with proteins has been observed by many investigators. It has been found in general that changes in the dye molecule can make large changes in binding abilities. Similarly it has been noted that modifying the protein also showed marked changes in the protein-dye complexes. Workers have shown that differently charged anionic dye molecules disclosed distinctive binding abilities with bovine serum albumin (BSA).

It was the purpose of this research to see if some relationship could be drawn between the binding sites on the protein bovine serum albumin and the methyl reds by observing the spectral and binding changes of the protein-dye complexes and the competitive affect of their aminobenzoate ion analogs.

Shifts in the maximum absorption peaks were observed for the protein-dye complexes of the ortho- and para-methyl reds. Competitive effects of the aminobenzoate ions corresponding to the dye involved tend to return these peaks to the original absorption peak of the dye. However, it was observed that a shift of the absorption peak of the meta-methyl red-protein complex and competitive effect of the amino-benzoate ion analog was reversed. It can be concluded from these observations that the meta-methyl red is bound differently than either its ortho or para isomer.

Competitive studies of the methyl reds with aminobenzoate ions tend to further support the possibility that the ortho-methyl red may be bound differently than the meta-methyl red. It was found that in the case of ortho-methyl red-protein complex the ortho-aminobenzoate ion was the better competitor whereas in the meta-methyl red-protein complex

it was the poorest competitor. With respects to the para-methyl red-protein complex, all three aminobenzoate ions were of equal strength in their competitive ability.

The quantitative determinations of the extents of binding of each of the aminobenzoate ions using the p-diethylaminobenzaldehyde reagent were made at the wavelengths for maximum absorption of the resulting Schiff's bases. The binding by BSA revealed that the ortho-aminobenzoate ion was bound in the largest quantity followed by meta- and para-aminobenzoate ions respectively.

The degree of binding of the methyl reds with BSA is directly related to their K_a values and the same relationship was noted with their solubilities.

Since ortho-aminobenzoic acid is the only aminobenzoic acid that can engage in intramolecular hydrogen bonding, and since this has been shown to be a consideration which favors binding to BSA one should thus eliminate it in the comparison of the aminobenzoic acids. If this is done, then the order of binding of the remaining two isomers is directly related to their solubilities and K_a values.

Although no definite conclusions can be made for competitive studies of the aminobenzoate ions regarding the type of binding that occurs, it is suggested that the ortho-methyl red is bound differently than that of the meta-methyl red. There is also an indication that the para-methyl red may also be bound differently from either of these two isomers.